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(54) **Title:** METHOD FOR DISRUPTING BACTERIAL BIOFILMS AND PREVENTING BACTERIAL BIOFILM FORMATION USING A COMPLEX OF ANTIMICROBAL PEPTIDES OF INSECTS

(57) **Abstract:** The present invention relates to the fields of medicine, hygiene, cosmetology and veterinary and could be used for preventing bacterial biofilm formation and disrupting biofilms formed by pathogenic and opportunistic bacteria of various kinds at a skin and other surfaces of organism, surfaces of implants and medical instruments. Preferred application field of the invention is a prevention of forming biofilms resistant to antibiotic and antiseptic action, and a disruption of those biofilms. The invention is based on treating the infected surface with a specimen comprising a complex of antimicrobial peptides of *Calliphora vicina* (Diptera, Calliphoridae) or other species of synanthropic saprophages from the order Diptera, particularly, C. *vomitoria, Lucilia sericata, Musca domestica, Hermetia illucense*, in combination with an antibiotic or antiseptic. The antibiotic or antiseptic used for that end is selected from among aminoglycosides, beta-lactams, glycopeptides, macrolides, lincosamides, fluoroquinolones, amphenicoles, and tetracyclines or quaternary ammonium salts, or other antibacterial matters that exhibit a synergistic or additive effect with the complex of antimicrobial peptides. The new method allows for: reducing the antibiotic or antiseptic concentration needed for disrupting biofilms and preventing biofilm formation; decreasing an adverse aftereffect of bacterial infection chemotherapy; extending a list of antibiotics suitable for treating the biofilm infections, for disinfecting the implants, for the hygienic purposes, as well as changing from the systemic administration of antibiotic to the local administration of antibiotics directly to the nidus of infection.

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METHOD FOR DISRUPTING BACTERIAL BIOFILMS AND PREVENTING BACTERIAL BIOFILM FORMATION USING A COMPLEX OF ANTIMICROBAL PEPTIDES OF INSECTS

The present invention relates to the fields of medicine, hygiene, cosmetology and veterinary and could be used for disrupting pathogenic biofilms formed by bacteria of various kinds at a skin and other surfaces of organism, surfaces of medical products, implants and catheters.

A great variety of antibiotics and antiseptics being toxicant for bacteria and belonging to various classes of organic compounds (beta-lactams, macrolides, tetracyclines, fluoroquinolones, sulfonamides, amidoglycosides, imidasoles, compounds of peptide nature, ammonium quaternary salts, et al.) is used in medicine and veterinary. Most of antibiotics and antiseptics known at present are effective in the case of plankton (free-living) bacteria forms. When forming a biofilm (a multicellular bacterial community surrounded with a matrix and adhered to external or internal surfaces of host organism or inanimate things), bacteria gain a resistance to antibiotics and antiseptics [1, 2] and become inaccessible for destructing by immune system cells [3]. Therefore, treatment and prevention of diseases promoted by the biofilms is very difficult [4, 5]. It is known that biofilms induce about 80 % human bacterial infections [6], as well as various inflammatory diseases and also autoimmune and oncology diseases related thereto, cardiovascular system damages. As a result, the biofilms serves as one of the main causes of disease incidence and mortality all over the world. A high resistance of the biofilms to external actions creates problems for disinfecting the medical products, supporting the personal and professional hygiene, treating the farm animals and pets. Accordingly, a development of methods for disrupting bacterial biofilms is one of the most relevant problems of modern medicine, veterinary and related fields of health service.

Use of natural and synthetic antimicrobial peptides is considered as one of advanced trends for solving this problem [6-9]. However, the practical realization of that idea runs against a considerable difficulties related to poor efficiency and high cost in using those peptides, risk for developing the cross-resistance to human and animal endogenous antimicrobial peptides in bacteria, and many others [10-12]. In order for increasing the efficiency of the treatment, it is proposed to create combinations of antimicrobial peptides and conventional antibiotics, in which combinations the peptide and antibiotic show the synergistic effect on the biofilms. Particularly, it is proposed to use synthetic antimicrobial peptides in combination with the antibiotics of various groups for treating the infections caused by bacterial biofilms [13]. With the same aim, it is proposed to use the protein of bacteriophage lysine in combination with antibiotics [14]. This method for disrupting the biofilms (RU 2014149348 A, 05.09.2013 "Method for preventing, disrupting and processing a biofilm with bacteriophage lysine") is the closest technical solution to the claimed invention and is chosen as the prototype, where a peptide (protein) product of the protein synthesis is used as the antibiotic synergist. It is important to emphasize that both above inventions use an individual compound of peptide nature (synthetic peptide [13] or natural protein [14], and the method for disrupting a biofilm consists in use of that compound in combination with a certain antibiotic. Multi-component combinations to that end did not used until now because of the technical complexity for developing those combinations and high cost of their manufacture. Meanwhile, natural mechanisms of the genetic immunity of multicellular animals include, as is well-known, complex aggregates of antimicrobial peptides. This provides a variety of key advantages that are absent in individual antimicrobial peptides and antibiotics, and in particular, prevents the development of bacteria resistance [16].

The technical problem of the claimed invention consists in developing a method for disrupting biofilms using a cooperative or successive action of natural complexes of antimicrobial peptides and known antibiotics. The technical re-

sult of the invention consists in enhancing efficiency for treating human and animal diseases invoked by pathogenic biofilms. This result is achieved due to increasing the biofilm sensitivity to antibiotics under the action of complex of antimicrobial peptides, reducing the therapeutically effective concentration of antibiotic and, accordingly, the toxicity level thereof for a patient. In order for solving that problem, it is proposed to use purified natural complexes of antimicrobial peptides that are synthesized by cultures of insects of the order Diptera. The specimen being obtained is used cooperatively or successively with an antibacterial means (or with a combination of antibacterial means) selected from among antibiotics or antiseptics that are toxicant for the given bacterial species. Herewith, the antibacterial means are preferred that exhibit the synergistic effect with the complex of antimicrobial peptides when testing *in vitro* at biofilms of the given species.

The essence of the claimed invention

Known are two basic forms being specific for most bacteria: plankton form (free-living cells providing the infection of host organism) and biofilms (a multicellular community of one or more species of microorganisms dipped into the matrix released by those microorganisms, which community being adhered to various surfaces). Being a part of the biofilm, a bacterial colony can persist indefinitely long time in the host organism, forming, as and when necessary, plankton cells transmitting an infection out of the primary site [1-5].

The main means for treating bacterial infections, including biofilms, are antibiotics. However, as was already noted above, most of antibiotics possess a low or zero activity in respect of biofilms in comparison with the plankton form of the same strain. A wide spread of strains having a genetic resistance to antibiotics constricts to an even greatyк degree the possibility for treating the biofilm infections. For solving that problem, in sources of literature, antimicrobial peptides are proposed as the synergists enhancing the anti-biofilm activity of antibiotics [6-9,13,14]. The now existent estimates of synergist effects (the “checker-

board" method) allow for developing mainly the binary compositions (1 peptide + 1 antibiotic). This restricts essentially the possibilities of methods now available for disrupting bacterial biofilms. Particularly, such combinations have a narrow spectrum of therapeutic activity. Thus, the use of bacteriophage lysine [14] is applicable to only the biofilm formed by gram-positive bacteria of the type *Staphylococcus aureus* and not effective to gram-negative bacteria.

The claimed method for disrupting biofilms, as with the known methods, is based on a usage of the effect of synergism of the antimicrobial peptides and antibiotics. The key difference consists in that a purified complex of antimicrobial peptides of insects of the order Diptera is proposed to use as the antibiotic synergist instead of individual antimicrobial peptide (for example, the synthetic cationic peptide [13] or bacteriophage lysine [14]). This idea is based on researches of the authors of this invention in the field of immunology of insects, especially blowfly larvae *Calliphora vicina*. The authors have stated that the larvae of that type, in response to bacterial contamination, simultaneously synthesize and accumulate in hemolymph a complex of antimicrobial peptides that includes defensins, cecropins, diptericins, and proline-rich peptides [15, 16]. Some of those peptides damage selectively the cell wall of gram-positive (defensins) and gram-negative (diptericins, cecropins) bacteria, others disturb the synthesis of protein and RNA in a bacterial cell (proline-rich peptides). All four classes are typical for the immune system of insects of the order Diptera [15]. Accordingly, complexes of antimicrobial peptides obtained from various species could be used for embodying the claimed invention, as is shown by results of testing in the real time given in Examples 1 and 2. Besides the *C. vicina*, for that purpose could be used such species as *C. vomitoria*, *Lucilia sericata*, *L. caesar* (Diptera, Calliphoridae), Musca *domestica* (Diptera, Muscidae), *Hermetia illucense* (Diptera, Stratiomydae). Those species are united with such a peculiarity that all of them, as the *C. vicina*, belong to synanthropic saprophagous dipterans living in environments filled maximally with pathogenic microflora of human beings,

farm animals and pets. Therefore, the antimicrobial complexes of synanthropic saprophages exhibit high activity just in respect to microflora of that type [15-16]. The ecology peculiarities of the *C. vicina* and other above-listed species of saprophagous insects of the order Diptera allow for cultivating them on industrial scale at cheap feeding substrates, which makes the biosynthesis of the claimed antimicrobial complex as technically and economically realizable.

The unique characteristic of the complex of insect antimicrobial peptides as exemplified by *C. vicina* is a complexity of the composition thereof. The results of the conducted research, summarized in the Example 3, show that not less than 11 individual antimicrobial peptides participating in the immune response of that insect are present in the composition of that complex. This fact causes the *C. vicina* and allied species of the order Diptera especially advantageous for realizing the claimed method for disrupting biofilms and preventing biofilm formation. However, the evolutionary conservatism of natural immunity mechanisms allows to suppose that complex of antimicrobial peptideses of other organisms can also be used for realizing the claimed method.

It is known that the complex of antimicrobial peptides of the *C. vicina* is able for disrupting the biofilms formed by various bacteria types, but this requires for creating high concentrations of the complex (from 1.5 to 7.6 g/l depending on bacterial species) [18]. This fact limits essentially the possibility for using the complex in medicine and other areas. Theoretically, that limitation can be eliminated by means of combining the natural complex of antimicrobial peptides and antibiotics or antiseptics forming a synergist pair therewith. However, that supposition did not considered in the literature and did not subjected to experimental study. Respectively, methods for disrupting bacterial biofilms based on the use of the complex of antimicrobial peptides of the insects of the order Diptera in combination with other antimicrobial means are therefore unknown. Such a problem had been for the first time set and solved by the authors of the

present invention. Results of corresponding experimental studies are given in specific embodiments.

With this object in mind, the authors have researched an anti-biofilm activity of the complex of antimicrobial peptides (CAMP) from *C. vicina* in combination with antibiotics from the groups of aminoglycosides, beta-lactams, glycopeptides, macrolides, lincosamides, fluoroquinolones, amphenicoles, and tetracyclines, as well as antiseptics from the group of cetrimoniums (quaternary ammonium salts). Detailed description of the experiments is given in the Examples 4-5. The main technique for studying was an analysis of interaction of various antibacterial means in the *in vitro* system, which analysis is widely used in conducting analogous studies (the "checkerboard" technique). Biofilms resistant to antibiotics are used as a biological model, which biofilms being formed by bacteria *Escherichia coli*, *Staphylococcus aureus*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*. The biofilms formed by those bacteria cause the most of clinically significant bacterial infections. Data showing an effect of the complex of antimicrobial peptides onto the anti-biofilm activity of antibiotics and antiseptics are summarized in the Table 1. A value of $MBIC_{90}$ (an antibiotic concentration inhibiting the metabolic activity by 90% in the standard TTC test) served as a quantitative criterion of the anti-biofilm activity.

Among the studied 20 variants of CAMP combinations with antibacterial means of various classes, 8 combinations show an additive effect (FICI > 0.5), and 11 combinations show a synergist effect (FICI < 0.5). In only one case (polymyxin B at the biofilm of *E. coli*), an antibiotic effect potentiation was not indicated. Both types of interaction allow for disrupting a biofilm and decrease the therapeutically effective concentration of antibiotic. The obtained results of the experimental studies obviously demonstrate, prove and confirm 14 dependent claims of the independent claim 1 of the claimed method for disrupting biofilms. Several most advanced embodiments of the invention should be extracted depending on the type of biofilm.

Thus, the biofilm of the Staphylococcus aureus appeared absolutely insensible to the action of beta-lactam meropenem, aminoglycosides of amikacin and kanamycin ($MBIC_{90}$ > 500 μg/ml), lincosamide clindamicyn ($MBIC_{90}$ > 250 μg/ml). Those antibiotics exhibit high anti-biofilm activity in the presence of the CAMP ($MBIC_{90}$ < 0.1, 1.5, 3 and 12 μg/ml, respectively). Consequently, the claimed invention allows using those antibiotics for treating the most widespread group of bacterial infections, the biofilms of *S. aureus*. Enlargement of antibiotic range for treating this group of infections has especially significant importance in the case of contamination by the particularly dangerous methicillin-resistant (MRSA) and vancomycin-resistant (VRSA) forms of *S. aureus*. According to the obtained data, a combination of CAMP + aminoglycosides is promising for disrupting biofilms of MRSA, and CAMP + lincosamides – in the case of VRSA infection.

Some of antibiotics used in studying, being toxic for plankton cells of *S. aureus*, maintain the activity also in regard to biofilms of this species when applied in elevated concentrations. In this group, it should be especially mentioned the vancomycin and ampicillin, for which the CAMP serves as a powerful synergist permitting to decrease the concentration suppressing the biofilm of *S. aureus* from 38-24 to 1 μg/l and less. Expediency of using the combinations of the CAMP with those antibiotics for suppressing the biofilms of *S. aureus* seems to be evident. It should be noted that the lower values of amplification coefficient (C_a < 10) can provide a significant improvement in treating of *S. aureus* biofilm infection.

Thus, the benzalkonium chloride antiseptic being applied for disinfecting a wound surface, like other antiseptics, exhibits a high toxicity. The possibility to decrease a concentration thereof by 8 times, while retaining the anti-biofilm activity, allows to decrease the toxic effect onto tissues surrounding the wound and thus to improve a regimen of wound infection treatment.

A biofilm formed by another widespread pathogen, enterobacterium *E. coli*, exhibits a relative (although reduced in comparison with plankton cells of that bacterium) sensitivity to all studied antibiotics. Under these circumstances, a combination thereof with the CAMP provides a potentiation effect to all studied antibiotics. However, the especially high synergism level of the CAMP with such beta-lactams as meropenem and cefotaxime should be noted (reduction of the effective concentration more than 187 and 62 times, respectively). According to this index, the combination of the CAMP with meropenem and other beta-lactams seems to be especially promising. The combination of the CAMP with meropenem demonstrates also a synergistic action in regard to biofilms formed by other widespread pathogens such as *P. aeruginosa* and *A. baumannii*.

Combining the CAMP with antibiotics allows also providing another technically important result, namely reduction of the effective concentration of the CAMP. The best result from the viewpoint of disrupting the biofilm of *S. aureus* is exhibited by the combination of the CAMP with amikacin (reduction of the effective concentration more than 48 times), as well as with vancomycin, kanamycin and tetracycline (reduction of the effective concentration from 16 to more than 24 times). In regard to biofilms formed by other studied bacteria (*E. coli*, *P. aeruginosa*, *K. pneumonia*, *A. baumannii*), a combination with antibiotics of various classes allows also to reduce the effective concentration of the CAMP from 2 to more than 43 times depending on bacteria species and antibiotic type.

Apart from disrupting mature biofilms, the claimed method provides one more technically important result, namely deletion of free-living (plankton) cells of bacteria and, accordingly, preventing the biofilm formation by those cells (Example 6). A synergistic or additive action of the CAMP and antibiotics onto plankton cultures, while implementing the preventive measures, allows to reduce significantly the therapeutically effective concentration of antibiotic that is

necessary for preventing the biofilm formation, and thus to escape adverse consequences associated with applying high doses of antibiotic.

It should be noted that the complex of antimicrobial peptides and antibiotic can be included into one pharmaceutical composition and applied onto the biofilm surface simultaneously. Instead of this, antibiotic can be introduced into an organism, independently of specimen comprising the complex of antimicrobial peptides, parenterally, orally, or by any other method accepted in medicine and guaranteeing the contact of antibiotic with biofilm being disrupted.

The claimed method can be also realized by introducing the complex of antimicrobial peptides of insects in a composition of various medical devices (wound and burntreating coverings, catheters, implants, and so on) in order for disrupting the pathogenic biofilms and preventing the formation thereof.

Moreover, the claimed method can be realized by introducing the complex of antimicrobial peptides of insects in a composition of skin care cosmetic products in order to prevent skin damages caused by the biofilm formation.

It is also evident that the claimed method can be used in veterinary for treating animal bacterial infections similar to human diseases.

As a whole, the claimed method allows to comprehensively enlarge significantly the range of techniques used in medicine and related areas for treating bacterial infections, and to increase the efficiency thereof. The main advantages of the claimed method consist in the following:

1. A possibility for creating multi-component compositions due to using the natural complexes of antimicrobial peptides. As such complex, it is possible to use a combination of antimicrobial peptides of insects of the order Diptera, which combination comprising four different classes of peptides (defensins, cecropins, diptericins, proline-rich peptides) each of which is represented by several different forms. Now known methods for disrupting biofilms are limited with a scheme “one peptide plus one antibiotic” characterized by narrow spec-

trum of antibacterial activity and elevated risk of drug resistance development in bacteria.

2. A possibility for reducing the antibiotic concentration needed for disrupting biofilm due to potentiating (synergistic or additive) action of the complex of antimicrobial peptides of insects. This allows to reduce the therapeutical dose of antibiotic and, accordingly, a risk of developing adverse effects from applying thereof.

3. An enlargement of range of antibiotics applicable for disrupting biofilms. In particular, the results of scientific studies and concrete examples of testing have shown that beta-lactam antibiotics and aminoglycosides exhibit anti-biofilm activity in the presence of CAMP, and those two key groups of antibiotics are considered now of little avail for treating the biofilm infections. This is a new and promising line of research.

4. A change of antibiotic administration way. At the present time, majority of antibiotics are used systemically by parenteral or oral administration. Local administration of antibiotics is limited with insufficient clinical efficiency. In systemic administration, in order for creating a needed concentration in the nidus of infection, heavy doses of antibiotics are used, which induces a death of normal flora of the patient and a risk of another adverse events (renal toxicity, neurotoxicity, cardio toxicity, and so on). The claimed method allows increasing the efficiency of antibiotic while administering directly into nidus of infection (for example, by applying onto the biofilm surface) and thus can eliminate the necessity of the systemic administration thereof in some cases confirmed experimentally.

5. The studied antibiotics potentiate, in turn, the anti-biofilm action of the complex of antimicrobial peptides of insects, permitting to reduce the therapeutically effective concentration thereof and enlarging a possible application range.

6. At the present time, 19 combinations of the CAMP and antibiotics are experimentally justified with a confirmation of obtained results. It is practically

assured that this list will be from now forth significantly enlarged during the following science and experimental studies. The claimed method allows also varying a balance between therapeutical doses and administration ways of the CAMP and antibiotics.

7. Summarizing all of the aforesaid, the claimed method allows to enlarge essentially and sharply the possibilities of personalized treatment of bacterial infections taking into account the patient characteristics and the character of disease.

The results of numerous tests are confirmed by concrete exemplary embodiments given below.

Example 1

Production of specimen comprising a refined complex of antimicrobial peptides of insects of the order Diptera and analysis of the antibacterial activity thereof.

A technique for producing the specimen was corresponded to the procedure described earlier [16]. For that purpose, four species of insects from the order Diptera including three species of blowflies from Calliphoridae family *Calliphora vicina*, *C. vomitoria* and *Lucilia sericata*, and housefly *Musca domestica* from Muscidae family were used. The procedure for producing the specimen was as follows. Larvae were immunized by introducing a suspension of bacterial cells into the body cavity, and incubated for 24 hours. After termination of that period, the hemolymph was collected from larvae through a cut of cuticle and used for releasing the complex of antimicrobial peptides. For that purpose, the collected hemolymph was acidified with 0.1% trifluoroacetic acid (TFA), and insoluble precipitate was removed with centrifugation. The obtained supernatant fluid was applied at a column with C-18 sorbent pre-stabilized with 0.05% TFA (Waters, 35 CC SepPack cartridge), washed with 0.05% TFA and eluted with 50% acetonitrile / 0.05% TFA. The eluate was subjected to lyophilization and used in this one and following Examples as a purified complex of antimicrobial

peptides. The antibacterial activity of the complex was determined using a serial dilutions technique [19]. In three independent measurements, the mean values of the minimal inhibitory concentration (MIC) for plankton culture *E. coli* 774.1 are represented in the Table 2. All four complexes exhibited the expressed antibacterial activity. Herewith, the maximal activity had been found in the complex from *C. vicina*. This specimen had been chosen for following studies as the best embodiment of the invention.

Example 2

Antibacterial activity of specimen from Hermetia illucens

A specimen comprising a complex of antimicrobial peptides from *H. illucens* was produced according to the technique described in Example 1. The antibacterial activity of the complex was determined using a method of agar plates [19]. For that purpose, 7.5 ml of Luria-Bertany nutrient solution with agarose (Invitrogen) (bactotryptone 1%, yeast extraxt 0.5%, NaCl 1%) was embedded into sterile Petri dishes (diameter 9 cm). Prior to solidification, 2×10^5 cells of bacterial plankton culture of corresponding strain (Table 3) were introduced into the warm culture medium. A tested material was applied in a volume 2 μl at the surface of solidified culture medium. The dishes were incubated during 24 hours at +37°C, and a diameter of bacterial growth inhibition zone was measured. The sample from *C. vicina* was used as a reference one. Data from Table 3 show that, under conditions of the given experiment, the sample from *H. illucense*, unlike the sample from *C. vicina*, exhibited an activity in regard to *P. aeruginosa*. Therefore, the sample from *H. illucense* can have advantage in treating bacterial infection induced by the given bacterium.

Example 3

Compositional analysis of the complex of antimicrobial peptides

The sample comprising the complex of antimicrobial peptides from *C. vicina* was produced according to the technique described in Example 1. A

composition of the antimicrobial peptides was studies using the earlier described techniques such as liquid chromatography, mass spectrometry and transcriptome analysis [18]. A structure has been ascertained for 11 peptides responsible for the complex antimicrobial activity (Table 4). Among them, 5 peptides (Seq. ID Nos. 1, 2, 4, 9, 11) had been characterized earlier [18, 20], and other 6 peptides are new for science. Moreover, the composition includes antimicrobial peptides having molecular masses from 6773 to 6973 daltons, which structure is not deciphered now, and, probably, other minor components having the antimicrobial activity. In accordance with the classification accepted in a literature [21, 22], active combinations from *C. vicina* belong to four classes of insect antimicrobial peptides: defensins (Seq. ID No. 1), cecropins (Seq. ID Nos. 2, 3), diptericins (Seq. ID Nos. 4-8), and proline-rich peptides (Seq. ID Nos. 9-11).

Example 4

Disruption of biofilms formed by pathogenic bacteria when contacting with the CAMP from C. vicina and antibiotics of various classes. TTC test

In the investigation, strains *E. coli* ATCC25922, *S. aureus* 203, *P. aeruginosa* ATCC 27583, *K. pneumonia* 145, *A. baumannii* 28 were used, which strains having an enhanced capability for forming biofilms [18]. A technique for producing the biofilms was corresponded with the one described in that publication. Biofilms were produced in 96-well microplates. The wells were filled with a bacterial suspension having a cell concentration 5×10^5 CFU/ml, and that suspension was incubated during 24 hours at 37°C. The LB nutrient solution (Invitrogen) was used as the negative control. The sample of the *C. vicina* CAMP was prepared in accordance with the protocol described in Example 1. A modified cross-titration technique was used for studying an interaction at a biofilm between combinations of the CAMP from *C. vicina* and antibiotics. For that purpose, the 24-hour biofilm in the microplate was washed three times with 200 µl of the PBS solution and dessicated. Combinations of the CAMP from *C. vici-*

na and antibiotics were prepared in another 96-well microplate in such a way that two-fold dilutions of the sample were placed in horizontal rows of wells, and two-fold dilutions of antibiotics were placed in vertical rows of wells. Further, 100 μl of content from each well of that microplate were transferred to the microplate with a biofilm and incubated during 24 hours at 37°C. The biofilm formation was evaluated by staining thereof with tetrazolium chloride (TTC). For that purpose, by 11 μl of 0.2% TTC solution were added into all wells of the plate. After incubating during 1 hour at 37°C, OD_{540} were measured using a reader of the microplate Epoch (BioTek). The OD_{540} value of the 48-hour biofilm not subjected to antimicrobial compounds was taken as a control. All experiments were made in two replications. A minimal inhibitory concentration for biofilm (MBIC) was evaluated as $MBIC_{90}$, i.e., the sample concentration that suppressed the viability of 90% cells. Fractional inhibitory concentration index (FICI) was determined for each sequence of combinations according to the expression: FICI = FICA + FICB, where FICA is equal to minimal inhibiting concentration (MIC) of antibiotic A in combination with another antibiotic divided by MIC of the antibiotic A taken alone, and FICB is equal to minimal inhibiting concentration (MIC) of antibiotic B in combination with another antibiotic divided by MIC of the antibiotic B taken alone. The FICI was interpreted as follows: synergism at FICI < 0.5, additive effect at FICI $> 0.5 \leq 1$, no interaction at FICI $> 1 \leq 4$, and antagonism at FICI > 4. The anti-biofilm activity had been studied for total 1470 combinations of the CAMP and antibiotics using the TTC test.

The results are summarized in Table 5. The experiments with *S. aureus* showed that the combining with the CAMP has an expressed synergistic action on the anti-biofilm activity of aminoglycosides (amikacin and kanamycin), beta-lactams (ampicillin and meropenem), glycopeptide vancomycin, macrolide erythromycin, lincosamide clindamycin, chloramphenicol. The CAMP exerts the

additive effect onto an action of oxacillin and such antiseptic as benzalkonium chloride. Thus, the CAMP potentiates the action of all studied antibiotics and antiseptics at the biofilm from *S. aureus*. It has been established in experiments with *E. coli* that the CAMP exerts: a synergistic effect on disruption of this type biofilm by meropenem and cefotaxime, an additive effect on the efficiency of gentamycin, ciprofloxacin, chloramphenicol and tetracycline. It has been also established that the CAMP shows the synergism with meropenem when disrupting biofilm formed by *P. aeruginosa* and *A. baumannii*, and the additive effect at *K. pneumonia*. Among all studied 15 antibiotics and antiseptics of 9 various classes, the CAMP did not exerted the potentiating action only at the efficiency of polymyxin B.

Thus, the CAMP is practically a universal means for enhancing the anti-biofilm activity of antibiotics. The claimed method for disrupting biofilms with the combination of the CAMP plus antibiotic can be realized in a great number of combinations taking into account a biofilm type, a threshold for reducing the therapeutic dose of antibiotic and/or changing a method for administrating thereof to the nidus of infection.

Example 5

Disruption of biofilms formed by pathogenic bacteria when contacting with the CAMP from C. vicina and antibiotics of various classes. CV test

In this example, the interaction between the CAMP from *C. vicina* and antibiotics was studied using an alternative technique for analyzing the anti-biofilm activity – biofilm staining with crystal violet (CV test). The analysis technique was corresponded to the one described earlier [18]. Unlike the TTC test evaluating the effect in accordance with a level of reduction of cell metabolic activity, the CV test allows to evaluate a degree of biofilm disruption (thickness) in accordance with an amount of colorant bound by biofilm. A minimal inhibitory concentration of the CAMP, antibiotic or various combinations thereof served as an efficiency criterion, which concentration reducing the amount of

bounded CV by 90% in comparison with the control (BIC_{90}). 24-hour biofilms cultured in wells of 96-well plates were washed three times with 200 µl of sterile PBS solution and dried in the air. Sterile series were prepared by double dilutions of the CAMP and antibiotics in the PBS, 100 µl of each concentration were added in respective wells, and the plates were incubated during 24 hours at 37°C. Then, medium residues were removed, and the wells were washed three times with 200 µl of the PBS, dessicated in the air and stained during 2 min with 0.1% water solution of the CV (Lenreaktiv, Russia). The stained biofilms were washed three times with 200 µl of the PBS, dessicated in the air, and the colorant was diluted in 200 µl of 95% ethanol during 1 hour. Then, an optical density of the solution of the colorant bound by biofilm was measured at 570 nm wavelength at an instrument Epoch reader (BioTek). Each measurement was performed in two independent replications.

The obtained data are summarized in Table 6. As in the TTC test (Table 5), the combining with the CAMP enhanced the antibiotic activity as regard to biofilms of all studied strains of bacteria. Herewith, the synergism was enhanced in 8 cases and the additive effect – in two cases. The evaluations of the interaction types revealed in the TTC and CV tests have coincided in all testing examples. Thus, data of the CV test confirm the conclusion made on the basis of the TTC test results that the CAMP is a universal means for potentiating the antibiotic anti-biofilm activity. A comparison of results of the TTC and CV tests shows also that the claimed method for disrupting biofilms provides simultaneously a deletion of bacterial cells and disruption of biofilm components including the matrix. The last fact demonstrates one more important advantage of the proposed method – a possibility for accelerated removing the bacterial metabolism products and, accordingly, reducing the inflammatory and allergic responses accompanying the biofilm infections.

Example 6

Preventing the biofilm growth when contacting the bacteria plankton forms with the CAMP from C. vicina and antibiotics of various classes

Biofilms are formed by free-living (plankton) bacteria cells settling at surfaces of organism or nonliving objects. Deletion of the plankton cells in nidus of infection is the most reliable method for preventing the biofilm formation. At now, antibiotics and antiseptics are used for that end. The purpose of the experiments discussed in this Example was to ascertain the bactericidal action of the AMPV from *C. vicina* applied in combination with antibiotics and antiseptics onto the plankton cells of pathogenic bacteria. The strains *E. coli* ATCC25922, *S. aureus* 203, *P. aeruginosa* ATCC 27583, *K. pneumonia* 145, *A. baumannii* 28 all having a capability for forming biofilms [18] were used in those experiments. The plankton cultures were produced by incubating cells during night at 37°C in a liquid LB nutrient solution (Invitrogen). A cross-titration technique was used for studying an interaction between combinations of the CAMP from *C. vicina* and antibiotics in plankton culture. For that purpose, combinations of the CAMP from *C. vicina* and antibiotics were prepared in 50 μl of liquid nutrient solution in 96-well microplate in such a way that two-fold dilutions of the sample were placed in horizontal rows of wells, and two-fold dilutions of antibiotics were placed in vertical rows of wells. Further, 50 μl of bacterial suspension having a cell concentration of 106 CFU/ml were introduced in each well with the sample, and the plate was incubated during 24 hours at 37°C. The cell growth was evaluated by staining thereof with tetrazolium chloride (TTC). For that purpose, by 11 μl of 0.2% TTC solution were added into all wells of the plate. After incubating during 1 hour at 37°C, OD_{540} were measured using a reader of the microplate Epoch (BioTek). The OD_{540} value of the 48-hour suspension culture not subjected to antimicrobial compounds was taken as a control. All experiments were made in two replications. A minimal inhibitory concentration for plankton cul-

ture (MIC) was evaluated as MIC_{90}, i.e., the sample concentration that suppressed the viability of 90% cells.

The results are summarized in Table 7. The experiments with *S. aureus* has shown that combination with the CAMP has an evident synergistic effect on the antibiotic activity of chloramphenicol and tetracycline. The CAMP has an additive effect on action of amikacin, kanamycin, ampicillin, meropenem, vancomycin, erythromycin, clindamycin and benzalkonium chloride antiseptic. Thus, the CAMP potentitates the effect of almost all studied antibiotics and antiseptics at the plankton culture of *S. aureus*. It has been established in the experiments with *E. coli* that the CAMP has a synergistic effect on the activity of polymyxin and cefotaxime, an additive effect on the efficiency of ciprofloxacin, chloramphenicol and tetracycline. It has also been established that the CAMP exhibits a synergism with meropenem in suppressing the growth of planktonic cells of *A. baumannii* and an additive effect at *S. aureus*, *P aeruginosa* and *K. pneumoniae*.

Thus, the CAMP enhances significantly the bactericide effect of all studied antibiotics in regard to bacteria planktonic cells.

As the results of testing carried out in a real-time mode and under real conditions have shown, the claimed method allows for preventing the biofilm growth at an early stage of infection process prior to formation of mature biofilm, which fact has a significant science and practical importance for preventing, as was indicated above, the treatment of many diseases.

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Table 1. Influence of the complex of antimicrobial peptides of *C. vicina* (CAMP) on the anti-biofilm activity of antibiotics

Antibiotic	Class of antibiotic	Coefficient of amplification C_a ($MBIC_{90}$ of antibiotic/ $MBIC_{90}$ of antibiotic + CAMP)
S. aureus		
Meropenem	BL	>500
Amikacin	AG	>333
Kanamycin	AG	> 167
Vancomycin	GP	32
Ampicillin	BL	>24
Clindamycin	LA	>21
Oxacillin	BL	11
Chloramphenicol	AM	8
Benzalkonium chloride	BC	8
Erythromycin	ML	4
Tetracycline	TC	>1.5
E. coli		
Meropenem	BL	>187
Cefotaxime	BL	62
Gentamycin	AG	10
Ciprofloxacin	FQ	>3
Chloramphenicol	AM	>6
Tetracycline	TC	5
Polymyxin B	PM	1
P. aeruginosa		
Meropenem	BL	8
K. pneumonia		
Meropenem	BL	4
A. baumannii		

Meropenem	BL	16

AG – aminoglycosides, AM – amphenicols, BL – betalactams, FQ – fluoroquinolones, GP – glycopeptides, LA – lincosamides, ML – macrolides, PM – polymyxins, TC – tetracyclines, BC – benzalkonium chloride

Table 2. Antibacterial activity of preparations obtained from various insect species of the order Diptera

Producent species	MIC, mg/L (mean ± error of mean)
C. vicina	250 ± 0.0
C. vomitoria	420 ± 80
L. sericata	420 ± 80
M. domestica	2000 ± 0

Table 3. Comparative activity of C. vicina and H. Illucense antimicrobial complexes

Strain	Diameter of inhibition zone, mm	
	C. vicina	*H illucense*
M. luteus A270	19	14
E.coli D31	14	12
S. aureus 203	6.5	8
P.aeruginosa ATCC 27853	0	6.5

Table 4. Structure of antimicrobial peptides contained in C. vicina

Peptide	Molecular weight, Da (monoisotopic)	UniProt ID	Amino acid sequence
Defensins			
Seq ID № 1	4032.0	C0HJX7	*ATCDLLSGTGANHSACAAHCLLRGNRGGYCNGKA VCVCRN*
Cecropins			
Seq ID № 2	4156.0	C0HJX8	*GGWLKKIGKKIERVGQHTRDATIQGLAVAQQAAN VAATAR*
Seq ID № 3	6743.7		*MNFHKVFIFVALILAVFAGQSQAGWLKKIGKKIER VGQHTRDATIQGLAVAQQAANVAATARG*
Diptericins			
Seq ID № 4		C0HJX9	*DSKPLNLVLPKEEPPNNPQTYGGGGGSRKDDFDV VLQGAQXEV... (N-terminal)*
Seq ID № 5	11991.0		*MKFVYLLAISALCMAAMVKAQNKPFKLTLPKEEP KNLPQLYGGGGGSRKQGFDVSLGAQQKVWESQN KRHSVDVNAGYSQHLGGPYGNSRPAYNGGVGYTY KLVNDCTISG*
Seq ID № 6	4463.3		*DSKPLNLVLPKEEPKNLPQLYGGGGGSRK-DGFDVSLGAQQRV*
Seq ID № 7	7363.5		*NLPQLYGGGGGSRKDGFDVSLGAQQKVWESQNK RHSVDVNAGYAQHLSGPYGNSRPAYSGGASYTYRF G*
Seq ID № 8			*MNSFIFGNLCFSVAALAKADSKPLNLVLPKEEP-KNLPQLYGGGGGSRKDGFDVNLGAQQRVWE-SETNVIQ*
Proline-rich peptides			

Seq ID № 9		C0HJY0	*FVDRNRIPRSNNGPKIPIISNP... (N-terminal)*
Seq ID № 10	6205.2		*MCGKKFFFFVLMALMALTTQLASASPFVDRSRRP NSNNGPKIPIISNPPFNPNARP*
Seq ID № 11	*4442.2*		*SRDARPVQPRFNPPPPKRERPIIYDAPIRRPGPKT MYA*

Table 5. Effect of C. vicina CAMP preparation on anti-biofilm activity of antibiotics. TTC test

Antibiotic	**Strain**	**$MBIC_{90}$ antibiotic, mg/L**	**$MBIC_{90}$* anti-biotic + CAMP, mg/L**	**FICI ***	**Interaction type**
Amikacin	S. aureus 203	>500	1.5	0.087	Synergy
Kanamycin	S. aureus 203	>500	3.0	0.107	Synergy
Gentamycin	E. coli ATCC25922	6.25	0.6	0.763	Additive
Oxacillin	S.aureus 203	0.11	0.01	0.583	Additive
Ampicillin	S. aureus 203	24.0	<1.0	0.208	Synergy
Cefotaxime	E. coli ATCC25922	3.75	0.06	0.179	Synergy
Meropenem	S. aureus 203	>50	<0.1	0.168	Synergy
	E. coli ATCC25922	3.75	<0.02	0.297	Synergy
	P. aeruginosa ATCC 27583	19.0	2.4	0.28	Synergy
	K. pneumonia 145	9.4	2.3	0.583	Additive
	A. baumannii 28	18.8	1.2	0.247	Synergy
Vancomycin	S. aureus 203	38.0	1.2	0.165	Synergy
Erythromycin	S. aureus 203	9.4	2.4	0.422	Synergy
Clindamycin	S. aureus 203	>250	12.0	0.355	Synergy
Polymyxin B	E.coli ATCC25922	9.4	9.4	1	Индифферент-ность
Ciprofloxacin	E. coli ATCC25922	0.06	<0.02	0.542	Additive
Chloramphenikol	E.coli ATCC25922	3.0	<0.5	0.667	Additive
	S. aureus 203	6.0	0.75	0.417	Synergy

Benzalkonium chloride	S. aureus 203	12.0	1.5	0.749	Additive
Tetracycline	E. coli ATCC25922	0.8	0.15	0.708	Additive
	S. aureus 203	0.15	<0.1	0.749	Additive

* - the minimum values of $MBIC_{90}$ and FICI among the studied combinations of CAMP C vicina and the corresponding antibiotic

Table 6. Effect of a preparation containing a complex of antimicrobial peptides of C. vicina on the anti-biofilm activity of antibiotics of various classes. Crystal violet test

Antibiotic	Strain	$MBEC_{90}$* antibiotic, mg/L	$MBEC_{90}$** antibiotic + CAMP, mg/L	FICI**	C_a***	CAMP and antibiotic interaction type	
						Crystal violet test	TTC test (data from Table 5)
Meropenem	S.aureus 203	>50	<0.1	0.169	>500	Synergy	Synergy
	E.coli ATCC25922	1.88	0.03	0.349	62.7	Synergy	Synergy
	P.aeruginosa ATCC 27583	9.4	0.3	0.159	31.3	Synergy	Synergy
	A.baumannii 28	9.4	0.59	0.291	15.9	Synergy	Synergy
	K.pneumonia 145	6.25	1.17	0.687	5.3	Additive	Additive
Cefotaxime	E.coli ATCC25922	1.88	0.12	0.397	15.7	Synergy	Synergy
Amikacin	S.aureus 203	>250	<1.0	0.024	>250	Synergy	Synergy
Kanamycin	S.aureus 203	>500	4.0	0.137	>125	Синер-гизм	Синергизм
Ciprofloxa-cin	E.coli ATCC25922	0.06	<0.02	0.583	>3.0	Additive	Additive
Chloram-phenicol	S.aureus 203	2.0	<0.5	0.438	>4.0	Synergy	Synergy

*minimum biofilm eradication concentration

** - the minimum values of $MBEC_{90}$ and FICI among the studied combinations of CAMP C. vicina and the corresponding antibiotic

*** Coefficient of amplification ($MBEC_{90}$ of antibiotic/ $MBIC_{90}$ of antibiotic + CAMP)

Table 7. Effect of C. vicina CAMP on the activity of different classes of antibiotics against planktonic cells. TTC test

Antibiotic	**Strain**	$MBIC_{90}$ antibiotic, mg/L	$MBIC_{90}$* antibiotic + CAMP, mg/L	**FICI****	**Interaction type**
Amikacin	S. aureus 203	1.88	0.48	0.833	Additive
Kanamycin	S. aureus 203	3.1	<0.1	0.527	Additive
Ampicillin	S. aureus 203	0.045	0.006	0.542	Additive
Cefotaxime	E.coli ATCC25922	0.09	0.011	0.415	Synergy
Meropenem	S. aureus 203	0.023	<0.002	0.667	Additive
	P.aeruginosa ATCC 27583	0.96	0.48	0.516	Additive
	K. pneumoniae 145	0.012	<0.002	0.833	Additive
	A. baumannii 28	0.75	0.045	0.393	Synergy
Vancomycin	S. aureus 203	0.47	0.12	0.589	Additive
Erythromycin	S. aureus 203	0.94	0.03	0.589	Additive
Clindmycin	S. aureus 203	75	4.7	0.729	Additive
Polymyxin B	E.coli ATCC25922	2.4	<0.1	0.375	Synergy
Ciprofloxacin	E.coli ATCC25922	0.03	0.015	0.667	Additive
Chloramphenicol	E.coli ATCC25922	4.7	0.3	0.67	Additive
	S. aureus 203	4.7	0.6	0.418	Synergy
Benzalkonium chloride	S. aureus 203	0.3	0.15	0.75	Additive
Tetracycline	E.coli ATCC25922	0.47	0.03	0.729	Additive
	S. aureus 203	0.12	0.03	0.416	Synergy

* - the minimum values of MBIC90 and FICI among the studied combinations of CAMP C vicina and the corresponding antibiotic

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Claims

1. A method for disrupting bacterial biofilms and preventing bacterial biofilm formation, said method comprising a step of contacting bacteria with an complex of antimicrobial peptides of insects, said peptides including defensins, cecropins, diptericins and proline-rich peptides, in combination with antibiotics or antiseptics.

2. The method according to Claim 1, wherein the complex of defensins, cecropins, diptericins and proline-rich peptides being produced from insects from the order Diptera of the families Calliphoridae, Moscidae and stratiomyidae.

3. The method according to Claim 2, wherein the insects from the order Diptera pertain to species Calliphora vicina, Lucilia sericata, Musca domestica or Hermetia illucense.

4. The method according to Claim 3. wherein the complex of defensins, cecropins, diptericins and proline-rich peptides includes amino acid sequences SEQ ID Nos. 1-11 or variations thereof having at least 80% identity with homological domains defining the antimicrobial activity of those peptides.

5. The method according to Claim 1, wherein the complex of defensins, cecropins, diptericins and proline-rich peptides being produced by chemical or genetic engineering synthesis of amino acid sequences SEQ ID Nos. 1-11 or variations thereof having at least 80% identity with homological domains defining the antimicrobial activity of those peptides.

6. The method according to Claim 1, wherein the antibiotics or antiseptics being represented by one or more combination from among aminoglycosides, beta-lactams, glycopeptides, macrolides, lincosamides, fluoroquinolones, amphenicoles, and tetracyclines or quaternary ammonium salts.

7. The method according to Claim 6, wherein the antibiotic or antiseptic being chosen from among compounds which combination with the complex of

antimicrobial peptides of insects, said peptides including defensins, cecropins, diptericins and proline-rich peptides having a synergistic or additive effect at a process of disrupting bacterial biofilms or preventing bacterial biofilm formation.

8. The method according to Claim 1, comprising the step of contacting the complex of antimicrobial peptides of insects, said peptides including including defensins, cecropins, diptericins and proline-rich peptides with bacteria at a skin, mucous coat, wound surface, erosion or ulcer surface.

9. The method according to Claim 1, comprising the step of contacting the complex of antimicrobial peptides of insects, said peptides including including defensins, cecropins, diptericins and proline-rich peptides with bacteria at a surface of medical device, catheter or implant.

10. The method according to Claim 1, wherein the antibiotic or antiseptic being applied at a skin, mucous coat, wound surface, erosion surface or ulcer processed with the complex of defensins, cecropins, diptericins and proline-rich peptides of insects.

11. The method according to Claim 1, wherein the antibiotic being administered into an organism parenterally, orally, transdermally, through mucous coat, inhalation, or in the form of aerosol.

12. The method according to Claim 1, wherein the biofilm being formed by bacteria *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*.

13. The method according to Claim 1, wherein the complex of defensins, cecropins, diptericins and proline-rich peptides being a part of pharmacological composition designed for treating bacterial infections.

14. The method according to Claim 1, wherein the complex of defensins, cecropins, diptericins and proline-rich peptides being a part of medical device designed for applying at a skin, mucous coat, wound surface, erosion or ulcer surface.

15. The method according to Claim 1, wherein the complex of defensins, cecropins, diptericins and proline-rich peptides being a part of cosmetic products for a skin care.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/RU 2018/000346

A. CLASSIFICATION OF SUBJECT MATTER

A61K 38/57 *(2006.01)*
A61K 35/64 *(2015.01)*
A61K 8/64 *(2006.01)*
A61P 31/04 *(2006.01)*

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K 8/00, 8/64, 35/00, 35/64, 38/00, 38/57, A61P 31/00, 31/04

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PatSearch (RUPTO internal), Esp@cenet, PAJ, USPTO, Information Retrieval System of FIPS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	GORDYA Natalia et al. Natural antimicrobial peptide complexes in the fighting of antibiotic resistant biofilms: Calliphora vicina medicinal maggots. PLOS ONE, 2017, 12(3): e0173559, abstract, p. 2, lines 22-26, p. 4, lines 37-45, p. 5, lines 1-4, p. 13, last paragraph, p. 14, table 6, p. 15, lines 31-35, p. 16, lines 8-14, 20-27, 26-27	1-4, 6-7, 12-13 5, 8-11, 14-15
Y	RAHNAMAEIAN Mohammad et al. Short antimicrobial peptides as cosmetic ingredients to deter dermatological pathogens. Appl Microbiol Biotechnol, 2015, 99(21): pp. 8847-8855, abstract, p. 8850, right col., p. 8852, right col.	5, 8, 10, 15
Y	RU 2548786 C2 (NOVABIOTICS LIMITED) 20.04.2015, p. 9, lines 30-32, 47-48, p. 10, lines 1-2, p. 11, lines 5-8	9, 11, 14

☐ Further documents are listed in the continuation of Box C.

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Date of the actual completion of the international search	Date of mailing of the international search report
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